

## Article

# Improvement of Digestate Stability Using Dark Fermentation and Anaerobic Digestion Processes

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**Abstract:** This paper assessed the effect of dark fermentation, the fermentative phase in a two-stage anaerobic digestion system, in terms of digestate biostabilization efficiency. The digestates analyzed in this study were obtained from a pilot-scale system in which two different substrates were used in order to simulate both the digestion and co-digestion process. Biostabilization performances were evaluated by measuring the specific oxygen uptake rate (SOUR) of the outgoing digestates. This index allowed us to define the degree of effectiveness in terms of stabilization of organic matter, between the traditional anaerobic digestion process and the two-stage configuration. Considering the traditional process as a reference scenario, the results highlighted an increase in biological stability for the two-stage co-digestion process, consisting of a dark fermentation stage, followed by an anaerobic digestion one. Digestates biostabilization efficiency increased up from 6.5% to 40.6% from the traditional one-stage configuration to the two-stage one by improving the anaerobic digestion process through a preliminary fermentative stage. The advantages of the two-stage process were due to the role of dark fermentation as a biological pre-treatment. Considering the partial stability results related to the second stage, biological stability was improved in comparison to a single-stage process, reaching an efficiency of 42.2% and 55.8% for the digestion and co-digestion scenario respectively. The dark fermentation phase allowed for a higher hydrolysis of the substrate, making it more easily degradable in the second phase. Results demonstrated better biostabilization performances of the outgoing digestates with the introduction of dark fermentation, resulting in more stable digestates for both the digestion and co-digestion process.

**Keywords:** bio-waste; two-stage process; bio-hydrogen; bio-methane; bio-fuels; respirometric test; specific oxygen uptake rate (SOUR)

## 1. Introduction

In order to develop a sustainable-resource-efficient future and reduce environmental pressures such as greenhouse gas emissions, the European Union has promoted legislation that has had an impact on waste management, with the aim of closing the material loop [1]. Considering the Circular Economy Package Action Plan promoted by the European Union [1], waste generation should be minimized by increasing the value of products as much as possible, turning waste into resources for new purposes [2,3]. Most of the municipal waste produced in the EU is organic (about 39.8 million tons) [4], therefore the effectiveness of the treatment applied to organic substances is of fundamental importance [5]. In this context, anaerobic digestion (AD) is considered an interesting technology for bio-waste valorization, due to its potential to convert biodegradable materials into bio-fuels and bio-based products [6]. The effectiveness of anaerobic digestion is measured in terms

of the biological stability of the output materials. If an output is stable, it means that it is not able to generate further residual impacts, such as climate-changing gases and leachates. Therefore, the optimization of biological processes through the assessment of the biological stability of process outputs is becoming increasingly important [7]. Nowadays, a significant capacity of AD technology is in the wastewater treatment sector for the treatment of wastewater sludge. However, co-digestion of sludge and biodegradable waste has received more interest, because it represents a strategic approach in wastewater and waste management [8]. In fact, AD technology provides useful process outputs as biogas, a renewable source of energy that can be used in cogeneration plants as an alternative to fossil fuels [9], and also digestate, a residual organic matter with high fertilizer and organic amendment properties that can be applied in agriculture as a substitute for synthetic fertilizers [10].

From the perspective of process optimization, the production of bio-hydrogen through a two-stage AD process is considered to be a promising solution [11–15]. In the two-stage configuration, the traditional AD process is divided into two reactors connected in series to each other in dark conditions. In the first reactor, namely, the dark fermentation (DF) reactor, the fermentative phase is performed and a hydrogen-rich biogas is produced with a release of volatile fatty acids (VFAs) in the digestate. The methanogenic phase is carried out in the second reactor, where the residual organic matter and VFAs are converted into a methane-rich biogas [16]. In addition to splitting the AD process in two stages, another possibility for improving AD efficiency is the biological treatment of different biodegradable substrates simultaneously, called the co-digestion process, which contributes to energy production enhancement and nutrient recycling [8,17–19]. Food waste, in particular the organic fraction of municipal solid waste, and wastewater sludge, represent two-optimal substrates for the co-digestion process due to their wide availability in the municipality and their synergistic characteristics in providing an important contribution to the digestion process [20]. Previous studies have investigated the role of DF in a two-stage AD process in terms of bio-fuel production. The first fermentative stage can be seen as a biological pre-treatment step that improves biogas production in the following AD stage [16]. In fact, in addition to producing hydrogen-rich biogas, the DF stage contributes to improving the hydrolysis of the feedstock by supplying readily available organic matter into the AD reactor, resulting in an increase of methane production [21–25]. As regards anaerobic digestate and the influence that a two-stage AD process has on it, the information concerning digestates' biological stability is still limited and further investigation is needed in terms of biostabilization efficiency. To the best of the authors' knowledge, this work is the first study to assess the stability of DF digestates, in comparison to the traditional AD process.

Nowadays, the application of digestate in agriculture as organic fertilizer faces many barriers, due to different national fertilizer regulations within the European Union, which vary especially according to the feedstock treated in the AD process. Therefore, the EU Fertilizer Regulation aims to define a homogeneous regulatory framework in line with the circular economy model, to promote the use of fertilizers obtained from bio-waste recycling [26]. Based on that, digestates need to comply with chemical, physical and biological standards, depending on the feedstock used. Biological stability is a characteristic of digestate that should be considered in assessing the possibility of using it in agriculture as an organic amendment for balancing the soil humus content [27]. This feature defines the content of readily biodegradable matter in an organic matrix and its degree of decomposition and also allows for evaluating the treatment efficiency of AD plants in terms of environmental impacts reduction [28]. Stability is typically evaluated by respirometric methods, but there are no standard European procedures for its assessment and each country uses its own rules [29]. Among the several methods for measuring biological stability (self-heating test, Dynamic Respirometric Index), respirometric tests that measure the oxygen uptake rate during microbial respiration activity are considered the most suitable for its estimation [7,30]. The specific oxygen uptake rate (SOUR) test is considered a short, indirect and reliable aerobic assay to assess biological stability. It is a measure of oxygen consumption during microbial respiration activity of a solid matrix suspended in an aqueous solution. Oxygen dispersion in the liquid phase is guaranteed by means of a continuous stirring and

an intermitted aeration. The dissolution of the sample in a liquid state avoids the oxygen transfer limitation due especially to the structure and moisture content of the sample [31]. The bulk of the research has focused its attention on the evaluation of digestate stability of a traditional AD process and of other matrices in terms of cumulative oxygen demand in 20 hours ( $OD_{20}$ ). Tambone et al. [27,29–33] showed  $OD_{20}$  values for biologically untreated matrices i.e., ingestates and household organic waste of  $243 \pm 8 \text{ mgO}_2 \text{ g DM}^{-1} 20 \text{ h}^{-1}$  and  $233 \pm 39 \text{ mgO}_2 \text{ g DM}^{-1} 20 \text{ h}^{-1}$  respectively. Scaglia et al. [34] studied sewage sludge stability through the SOUR test, obtaining an average value of  $91 \pm 8 \text{ mgO}_2 \text{ g DM}^{-1} 20 \text{ h}^{-1}$  for untreated sludge and  $30 \pm 2 \text{ mgO}_2 \text{ g DM}^{-1} 20 \text{ h}^{-1}$  for anaerobically treated sludge. Orzi et al. [28] reported the results of the stability index of several ingestates and digestates coming from a full-scale thermophilic AD plant, which were on average equal to  $264 \pm 40 \text{ mgO}_2 \text{ g DM}^{-1} 20 \text{ h}^{-1}$  and  $95 \pm 16 \text{ mgO}_2 \text{ g DM}^{-1} 20 \text{ h}^{-1}$  respectively. Other results were reported by Tambone et al. [35] and Schievano et al. [36]. Nevertheless, information on the biological stability of the digestate obtained by a two-stage AD system is still limited.

Based on the above background, considering the influence of DF in biogas production, its effect on digestate stability should also be evaluated. The main purpose of this study is to compare the biological stability of the outgoing digestate obtained from a one-stage and two-stage AD system. Experiments were performed in semi-continuous pilot scale reactors, employing different substrates as feeding. Different scenarios were carried out in order to develop both the traditional AD process and the two-stage process, where the preliminary DF stage was added. Anaerobic performances were estimated in terms of digestate stabilization and evaluated through the SOUR test.

## 2. Materials and Methods

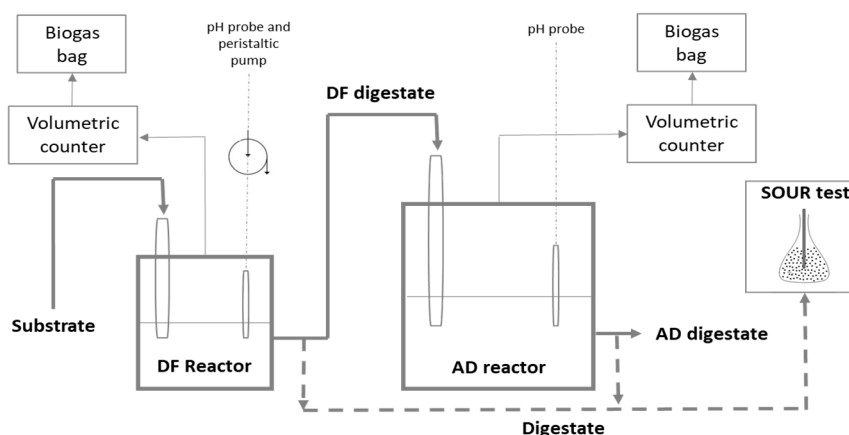
Experiments were carried out with two distinct feedstocks in order to perform both the digestion and co-digestion process. In addition, the tests were analyzed in two different configurations. For the evaluation of the conventional one-stage AD process, which represents the reference scenario, the methanogenic reactor was performed alone. Subsequently, the DF reactor was connected in series with the AD reactor with the aim of estimating the process in a two-stage system. In the study of Baldi et al. [21], a detailed description of the experimental set-up is shown, as well as the operational conditions for the continuous stirred tank reactors (CSTR) pilot-scale tests.

### 2.1. Feedstock and Digestate

Food waste (FW) and wastewater sludge (WWS) were used as substrates in the experimental tests, both separately and mixed together, with the aim of simulating a digestion or co-digestion system. The FW was collected by sampling about 10 tons of source-separated organic waste coming from a kerbside collection system in a Tuscan municipality (Italy). The sample was immediately treated in a food processor (Philips, Problend 6, Amsterdam, The Netherlands) for five minutes at maximum speed, diluted with tap water and stocked in a freezer at  $-4 \text{ }^{\circ}\text{C}$  until use. Concerning WWS, around 200 liters of aerobic sludge from the aerobic unit of a municipal WWTP were collected. The sample was stored in plastic tanks and refrigerated at  $4 \text{ }^{\circ}\text{C}$  until use. In order to depict a wet digestion technology, the food waste sample was diluted with the aim of achieving a slurry, with a total solid (TS) content of 5% by weight. Therefore, the thawed FW slurry was mixed with tap water or WWS to simulate, respectively, the digestion and co-digestion trials. In both experiments, the ratio of FW to water or WWS was about 1:5 by weight [21].

The digestate was taken from a pilot-scale system made of stainless steel (AISI 316) reactors operating under mesophilic conditions ( $37 \text{ }^{\circ}\text{C} \pm 1$ ). The one-stage and two-stage AD processes were carried out by means of two continuous stirred tank reactors (CSTR) adopted for performing the fermentative and methanogenic stages in dark conditions. The fermentation stage (DF) was evaluated using a 6 L reactor, while for the methanogenic stage a 20 L reactor was used (working volume of 3 L and 12 L respectively for DF and AD). The reactors were fed daily with feedstock through a syringe, with a capacity of 200 mL, and subsequently, the same amount of digestate was collected. In

the traditional one-stage configuration, the methanogenic reactor ran alone and therefore only the digestate coming from the AD reactor was sampled. In the two-stage process, the DF and AD were connected in series and only the outgoing digestate of the DF reactor was turned into the substrate for the subsequent AD tank. Two different digestates were collected in this configuration: the outgoing digestate of both the DF and AD reactors (Figure 1). After sampling, the digestate was centrifuged at 35,000 rpm for 30 minutes (Beckman Coulter, Allegra 25R Centrifuge, Brea, California, USA) and the solid phase was then stored in a freezer at  $-4\text{ }^{\circ}\text{C}$  until analysis.



**Figure 1.** Schematic representation of experimental set-up.

Summarizing, the substrates characterized by the sole FW (S1) and the mixture of FW and WWS (S2), were used respectively as feeding in the digestion and co-digestion configurations. The other samples constituted the outgoing digestates obtained from the DF and AD reactor. Table 1 shows the analyzed samples, their origins and time of process for treatment.

**Table 1.** Feedstocks and digestates samples studied. Process time depends on the operational condition, which is based on maintaining an organic loading rate (OLR) in the anaerobic digestion (AD) reactor, equal to  $2.5\text{ kgTVS/m}^3 \times \text{d}$ , as defined in the study of Baldi et al. [21].

Process	Origins	Digestion (FW)		Co-Digestion (FW + WWS)	
		Sample	Process Time (days)	Sample	Process Time (days)
-	Substrate	S1	0	S2	0
One-stage	AD digestate	S1.1_AD	17	S2.1_AD	17
Two-stage	DF digestate	S1.2_DF	3	S2.2_DF	3
	AD digestate	S1.2_AD	12.8	S2.2_AD	11.9

Each experimental trial was performed during the steady-state condition that corresponds to a period of three HRTs of anaerobic digestion reactor.

Analytical characterization of the substrates and digestates were performed in order to provide a detailed description of the media. Results are shown in Table 2.

**Table 2.** Feedstocks and digestates characterization. Averages and standard deviations are presented (n = 2).

Parameters	FW	WWS	S1.1_AD	S1.2_DF	S1.2_AD	S2.1_AD	S2.2_DF	S2.2_AD
pH	3.80 ± 0.00	7.10 ± 0.00	6.04 ± 0.30	5.22 ± 0.26	7.61 ± 0.38	7.30 ± 0.37	5.84 ± 0.29	7.29 ± 0.36
TS (% w/w)	12.75 ± 10.11	2.10 ± 0.20	4.00 ± 1.00	4.00 ± 1.00	2.00 ± 1.00	2.00 ± 1.00	4.00 ± 1.00	2.00 ± 1.00
TVS (% w/w)	10.93 ± 7.78	1.56 ± 0.40	1.10 ± 0.10	0.80 ± 0.10	0.70 ± 0.10	0.60 ± 0.10	1.00 ± 0.10	0.90 ± 0.10
TOC (%C w/w)	5.95 ± 5.73	1.20 ± 0.20	1.90 ± 0.29	1.67 ± 0.25	0.67 ± 0.10	0.70 ± 0.20	1.60 ± 0.20	0.60 ± 0.20
Ammonia (mgN/L)	580 ± 550	341 ± 47	763 ± 110	540 ± 81	1429 ± 210	783 ± 78	404 ± 40	721 ± 72
Acetic acid (mg/Kg)	1639 ± 963	830 ± 120	< 25	2429 ± 364	< 25	< 25	< 25	145 ± 25
Propionic acid (mg/Kg)	< 40	390 ± 71	499 ± 76	1371 ± 204	< 25	< 25	210 ± 31	118 ± 25
Butyric acid (mg/Kg)	-	-	98 ± 15	2846 ± 440	< 25	< 25	625 ± 93	< 25
Valeric acid (mg/Kg)	-	-	925 ± 140	3251 ± 480	< 25	< 25	1043 ± 160	< 25
C (% TS)	40.20 ± 5.94	58.90 ± 4.30	46.60 ± 7.00	48.00 ± 7.20	37.30 ± 5.60	35.00 ± 5.10	40.75 ± 6.25	28.00 ± 4.50
H (% TS)	6.65 ± 1.20	6.40 ± 0.50	6.90 ± 1.00	7.40 ± 1.10	5.25 ± 0.79	4.50 ± 0.50	5.50 ± 0.75	3.5 ± 0.50
N (% TS)	3.15 ± 0.35	7.50 ± 0.90	4.44 ± 0.67	4.80 ± 0.72	10.90 ± 1.60	9.00 ± 1.00	4.75 ± 0.50	3.50 ± 0.50
C: N	12.74 ± 0.46	7.85 ± 1.31	10.00 ± 1.50	10.00 ± 1.50	3.00 ± 0.45	4.00 ± 0.60	9.00 ± 1.40	9.00 ± 1.40
Ca (mg/L)	2660 ± 2,178	703 ± 85	729 ± 100	773 ± 100	591 ± 100	415 ± 100	631 ± 100	557 ± 100
Mg (mg/L)	378 ± 299	109 ± 25	100	103 ± 100	115 ± 100	109 ± 100	133 ± 100	153 ± 100
Na (mg/L)	945 ± 559	121 ± 27	3087 ± 250	1224 ± 100	923 ± 100	417 ± 100	1500 ± 230	1718 ± 260
P (mg/L)	383 ± 180	84 ± 21	147 ± 22	193 ± 28	200 ± 30	119 ± 17	155 ± 23	158 ± 23
S (mg/L)	191 ± 90	189 ± 86	90 ± 50	81 ± 50	67 ± 50	141 ± 50	100 ± 50	214 ± 50
Proteins (% w/w)	2.45 ± 2.05	0.90 ± 0.10	0.80 ± 0.10	0.70 ± 0.10	0.30 ± 0.10	0.70 ± 0.10	1.00 ± 0.20	1.00 ± 0.20
Lipids (% w/w)	2.10 ± 2.55	0.30	0.19 ± 0.02	0.43 ± 0.07	0.04 ± 0.01	0.01 ± 0.01	0.17 ± 0.03	0.03 ± 0.01
Carbohydrates (% w/w)	4.70 ± 3.82	0.10	1.10 ± 0.10	1.30 ± 0.20	0.30 ± 0.10	0.10 ± 0.10	0.90 ± 0.10	0.10 ± 0.10
Cellulose (% w/w)	2.25 ± 1.06	0.10 ± 0.00	0.30 ± 0.03	0.50 ± 0.10	< 0.10	0.10 ± 0.10	0.30 ± 0.10	0.10
Lignin (% w/w)	2.60 3.25	0.30 ± 0.00	0.30 ± 0.10	0.30 ± 0.10	0.10 ± 0.10	0.30 ± 0.10	0.50 ± 0.10	0.60 ± 0.10

## 2.2. Biological Stability Index: SOUR Determination

In accordance with the purpose of this study, the biological stability of each digestate was assessed through the evaluation of the SOUR index in order to evaluate the AD process efficiency in terms of stabilization improvement. The SOUR index was measured for each substrate and digestate collected during the experimental test, according to the methods proposed by Lasaridi and Stentiford [37] and modified by Adani et al. [38].

To perform the SOUR test, a 2.5 g (wet weight) sample was defrosted and taken, diluted with 500 mL of distilled water and mixed for approximately 30 s by means of a food blender (Problend 6, Philips, Amsterdam, The Netherlands). After mixing, the solution was further diluted with another 500 mL of distilled water and placed in a 1-liter bottle. To ensure optimal nutrient and pH conditions for process development, buffer and nutrients solutions were introduced in the aqueous blend. In particular,  $\text{MgSO}_4$ ,  $\text{CaCl}_2$  and  $\text{FeCl}_3$  solutions were made in accordance with the standard procedures for the biological oxygen demand (BOD) test [39] and 5 mL of each were added to the bottle. Furthermore, 1.1 mL of ATU (allylthiourea) and 15 mL of phosphate buffer solution were added to avoid nitrogenous oxygen demand and maintain a neutral pH value of the order of 7.0. The flask was placed on a magnetic stirrer (Velp Scientifica, AREX Digital PRO, Monza Brianza, Italy) which allows for a continuous mixing (250 rpm) and a constant solution temperature of 30 °C. The aqueous suspension was intermittently aerated through a fish-tank air pump following the sequence of aeration/reading of 20 min/15 min, in order to supply the oxygen consumed by the biomass during the test. During the reading period, a dissolved oxygen (DO) probe (Mettler Toledo, InPro6000, Optical  $\text{O}_2$  Sensors, Columbus, OH, USA) measured changes in the DO concentration in the solution. The signals coming from the DO probe were acquired by an automatic data acquisition system (LabView, National Instruments, Austin, TX, USA) that also controls the air pumping system.

The SOUR measure ( $\text{mg O}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$ ), represents the maximum oxygen consumption rate recorded during the overall experimental test. The SOUR value was calculated by the DO concentration drops measured during the reading cycle, using the following equation and according to the raw DO data analysis proposed by Lasaridi and Stentiford [40]:

$$\text{SOUR} = \frac{|\text{Smax}| \times V}{m \times \text{TS} \times \text{TVS}} \quad (1)$$

$|\text{Smax}|$  is the maximum slope of DO consumption expressed in absolute terms ( $\text{mg O}_2 \times \text{l}^{-1} \times \text{h}^{-1}$ ) and measured during the experimental period,  $V$  is the volume of the aqueous suspension (l),  $m$  is the wet mass of the sample (g),  $\text{TS}$  and  $\text{TVS}$  are, respectively, the total solids (wet basis) and total volatile solids (dry basis) content expressed in decimal fraction.

The respirometric test was performed over an experimental period of 20 hours and the cumulative oxygen demand ( $\text{OD}_{20}$ ) was also evaluated. The  $\text{OD}_{20}$  is calculated (using Excel) as the integral expressed by the subtended area of the  $\text{O}_2$  consumption curve obtained from the same data used to determine the SOUR index [40].

The biodegradability test was carried out in duplicate for each sample.

## 2.3. Analytical Methods

Each feedstock and digestate was monitored during the overall experimental period and analytically characterized. TS and TVS content and pH were measured according to standard methods [41]. Total organic carbon (TOC) was obtained as reported in EN ISO 13127 [42], ammonia and VFAs were measured as stated according to APHA [43] and US EPA [44] respectively. C, H, N content were obtained following EN 15407 [45] and Ca, Mg, Na, P and S were measured according to EPA 6010 D [46] and EN 13657 [47]. Finally proteins, lipids, cellulose and carbohydrates were measured in accordance with the European Commission regulations 2009/152/EC [48]. Lignin content was determined with the method proposed by Martillotti et al. (1987) [49].



## 2.4. Statistical Analysis

In order to verify the agreement between the SOUR and OD<sub>20</sub> results, the Welch's *t*-test was applied to each of the tested configurations to identify significant differences between the AD statistical populations. The comparison was made in pairs between the input feedstocks (S1 and S2), in the one-stage and two-stage AD processes.

## 3. Results and Discussion

### 3.1. Feedstock and Digestate Characterization

In order to provide a detailed description of the media, an analytical characterization of the feedstocks and digestates was carried out. The results are reported in Table 2 in terms of averages and standard deviations.

Based on the above results, the pH values were in the optimal range for the implementation of the AD process. In particular, it can be observed that in the fermentative reactor (S1.2\_DF and S2.2\_DF) the pH was in the acidic range of 5–6, which is optimal for the development of dark fermentation, according to the literature [12,50,51]. Referring to the methanogenic reactor, pH was measured around neutral values, typical of a conventional digestion process [52].

The analytical characterization of the media suggests a decrease of organic matter content during the AD digestion process. In fact, considering the content of TVS and TOC, it can be seen that these parameters drop during both the digestion and co-digestion processes. The organic matter content will be discussed in more detail in the following section, in terms of biological stability through the assessment of the stability index.

As concerned the macromolecules, carbohydrates were the main element of FW, unlike proteins, which were the main component of WWS. The carbohydrates and proteins contents measured in the feedstocks were lower than other findings in the literature [53,54], which is due to the dilution carried out in this work. Regarding the macromolecules content in the digestates, the results showed a significant reduction, especially for the digestion configuration, due to the degradation of the matrix during the AD process.

Concentrations of light metals, such as calcium, magnesium, sodium, potassium and sulfur are important for the improvement of the DF and AD processes, although considerable contents of these may inhibit the process behavior [55]. Considering the inhibitory threshold of metal ions in the feedstocks, as recorded in previous studies [56,57], the light metals contents of the feedstocks analyzed were measured below these values. In addition, ammonia content is considered another parameter that may inhibit the process. In fact, a high concentration of ammonia (above 1500 mg/L) could cause toxicity to the anaerobic bacteria and therefore inhibit methane production [4]. Several inhibitory thresholds were determined in the literature [58,59]. However, ammonia content of FW and WWS used in this study was found in a range that prevents process inhibition.

The results regarding acids highlight a significant production of VFAs during the dark fermentation phase. This result indicates that a proper hydrolysis process was developed in the fermentative stage with the release of VFAs in the liquid solution [25]. Therefore, VFAs concentration in the DF digestate of the two configurations was significant. A high content of VFAs is also evaluated for the AD digestate related to the digestion process of the sole FW. This result is correlated with the operational condition used to perform the experimental tests. To meet the experimental requirement, comparing the scenarios and ensuring the same conditions, a fixed OLR for the methanogenic reactor was maintained in each configuration. However, this fact determined that the HRT of the methanogenic reactor in a one-stage configuration was not sufficient for completing the degradation process, resulting in high VFAs and organic matter content.

As regards the digestates obtained from the co-digestion process, P<sub>2</sub>O<sub>5</sub>, K<sub>2</sub>O and CaO content of the organic matrices was also measured according to the EU Fertilizer Regulation in order to assess fertilizer and amendment properties. The high concentration of these parameters compared to the

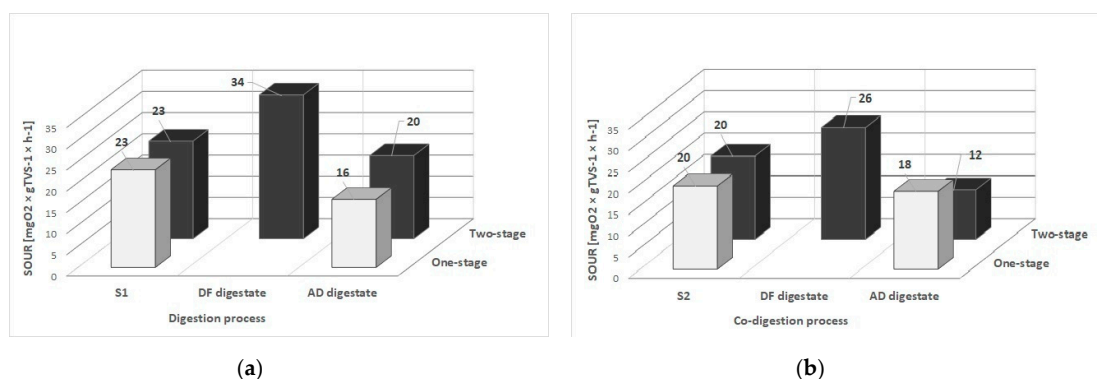
limits set by the EU Regulation confirms the optimal fertilizer and amendment characteristics of the digestate.

### 3.2. Biological Characterization

The average results of biological stability obtained by measuring the SOUR and OD<sub>20</sub> indexes are reported in Table 3. Figure 2 shows the SOUR indexes in the different steps of the AD configuration analyzed for both the digestion and co-digestion process. The trend of the SOUR index during the different phases of the process indicates the stabilization efficiency of the process through the evaluation of biostabilization parameters. For this reason, the SOUR index was evaluated for each sample collected during the experimental tests.

**Table 3.** Specific oxygen uptake rate (SOUR) and OD<sub>20</sub> indexes. Results are reported as averages and standard deviation (n = 2). The apex letters indicate the result of the statistical analysis performed with Welch *t*-test (values are not statistically different when *p* > 0.05). The same letters indicate that the populations are not statistically different.

Digestion (FW)			
Process	Sample	SOUR [mg O <sub>2</sub> × gTVS <sup>-1</sup> × h <sup>-1</sup> ]	OD <sub>20</sub> [mg O <sub>2</sub> × gTVS <sup>-1</sup> × 20 h <sup>-1</sup> ]
-	S1	23 ± 13 <sup>a</sup>	252 ± 92 <sup>c</sup>
One-stage	S1.1_AD	16 ± 6 <sup>a</sup>	107 ± 51 <sup>c</sup>
Two-stage	S1.2_DF	34 ± 11	320 ± 77
	S1.2_AD	20 ± 5 <sup>a</sup>	133 ± 59 <sup>c</sup>
Co-Digestion (FW + WWS)			
Process	Sample	SOUR [mg O <sub>2</sub> × gTVS <sup>-1</sup> × h <sup>-1</sup> ]	OD <sub>20</sub> [mg O <sub>2</sub> × gTVS <sup>-1</sup> × 20 h <sup>-1</sup> ]
-	S2	20 ± 1 <sup>a</sup>	156 ± 28 <sup>c</sup>
One-stage	S2.1_AD	18 ± 0 <sup>a</sup>	112 ± 21 <sup>c</sup>
Two-stage	S2.2_DF	26 ± 4	244 ± 25
	S2.2_AD	12 ± 0 <sup>b</sup>	135 ± 12 <sup>c</sup>



**Figure 2.** Stability index trend during one-stage and two-stage AD systems. Digestion process (a) and Co-digestion process (b) are reported.

SOUR values were assessed for the substrates used in the two configurations analyzed, that is, S1 for the digestion of the sole FW and S2 for the co-digestion of FW and WWS. Based on the results presented in Table 3 and Figure 2, the mean SOUR values of these samples were quite similar and equal to  $23 \pm 13 \text{ mgO}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$  and  $20 \pm 1 \text{ mgO}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$  for S1 and S2 respectively, corresponding to an OD<sub>20</sub> index of  $252 \pm 92 \text{ mgO}_2 \times \text{gTVS}^{-1} \times 20 \text{ h}^{-1}$  for S1 and  $156 \pm 28 \text{ mgO}_2 \times \text{gTVS}^{-1} \times 20 \text{ h}^{-1}$  for S2. As mentioned in the section on feedstock and digestates, S1 was obtained by diluting FW with tap water and S2 with WWS. Since the feeding slurry was to have a TS content of 5% to simulate a wet AD process, in S2, the percentage of FW was less than S1. WWS is an organic



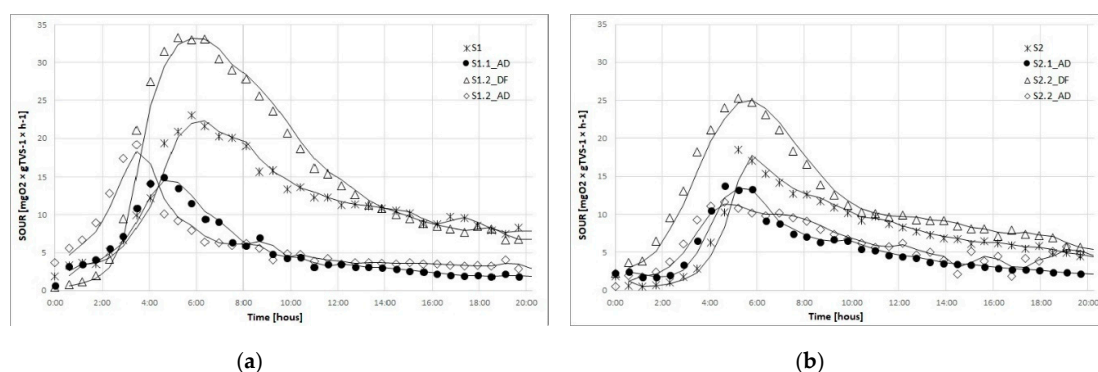
matrix already partially biodegraded during the aerobic treatment in the WWTP. Therefore, the organic carbon available within S2 was slightly lower than S1, which resulted in lower SOUR values. These values are consistent with Adani et al. [38] who found SOUR values for organic matrices sampled from a full-scale process of  $19 \text{ mgO}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$ . Schievano et al. [36] and Tambone et al. [27] studied different organic raw materials for the AD process obtaining an average range for  $\text{OD}_{20}$  values of  $30\text{--}410 \text{ mgO}_2 \times \text{gTS}^{-1} \times 20 \text{ h}^{-1}$ .

The biological stability test on the collected matrices was assessed when the steady state of the process was reached. S1.1\_AD and S2.1\_AD represent the digestate take from the methanogenic reactor during one-stage configuration. In both one-stage configurations, the SOUR index decreased for the outcoming digestate to  $16 \pm 6 \text{ mgO}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$  and  $18 \pm 0 \text{ mgO}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$  respectively for S1.1\_AD and S2.1\_AD, meaning that biological stabilization improved. The corresponding cumulative oxygen demand values were, respectively,  $107 \pm 51 \text{ mgO}_2 \times \text{gTVS}^{-1} \times 20 \text{ h}^{-1}$  and  $112 \pm 21 \text{ mgO}_2 \times \text{gTVS}^{-1} \times 20 \text{ h}^{-1}$  in line with the literature amounts [27,33,34].

As concerned the two-stage configuration, the SOUR index was evaluated for both the DF and AD digestates. Regarding the digestate coming from the DF reactor, it can be observed that the stability index decreases and therefore the SOUR values increase. The higher SOUR values obtained for the DF digestates output, equal to  $34 \pm 11 \text{ mgO}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$  and  $26 \pm 4 \text{ mgO}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$  for S1 and S2 respectively, were related to the greater production of VFAs in the first DF reactor. The complex molecules present in the substrate, such as carbohydrates, proteins and lipids, were degraded in this first stage and reduced to simpler molecules. This preliminary treatment improves the presence of available biodegradable substrate in the DF digestate which reduces the biological stability measured at this intermediate stage with SOUR values of  $34 \pm 11 \text{ mgO}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$  and  $26 \pm 4 \text{ mgO}_2 \times \text{gTVS}^{-1} \times \text{h}^{-1}$  respectively for S1.2\_DF and S2.2\_DF. After the preliminary phase, the DF digestate is turned into the feeding substrate for the AD reactor and the available biodegradable matter is then stabilized by the methanogenic bacteria which result in a reduction of the biological stability index.

Concerning statistical analysis, it can be observed that only in the two-stage co-digestion configuration the SOUR value of the AD digestate is statistically different from other populations. Welch test results confirm that DF in co-digestion introduces a significant modification in terms of process stability. The other populations are not statistically different, as indicated by the same apex letter in Table 3.

For a better evaluation of the SOUR index behavior during each step of the digestion and co-digestion process, Figure 3 shows the trend of the stability index during the two experimental configurations.



**Figure 3.** SOUR trend during the two configurations studied: digestion process (a) and co-digestion process (b).

As expected, the maximum rate of oxygen demand (represented by the SOUR value) is achieved within 2 to 8 h of the experiment period. The time required to get to the maximum depends on the stability of the materials, typically characterized by an oxygen demand curve with an initial lag

phase and a subsequent exponential increase before reaching the maximum [40]. In Figure 2 it can be observed that microbial respiration for S1.2\_DF and S2.2\_DF reaches maximum SOUR after rapid exponential growth and has the highest peaks due to the available biodegradable substrate. The other samples show a similar SOUR trend.

### 3.3. Effect of Dark Fermentation on Digestate Stability

The influence of DF on the AD process by splitting the traditional process into two phases has shown an enhancement in biogas production and methane content. As reported in the study of Baldi et al. [21], an increase in specific gas production in the two-stage digestion and co-digestion process was assessed compared to the traditional AD process. In particular, the specific methane production in the two-stage system improved from  $453.1 \pm 28.2 \text{ NLCH}_4 \times \text{kgTVS}^{-1} \times \text{d}^{-1}$  to  $482.1 \pm 24.0 \text{ NLCH}_4 \times \text{kgTVS}^{-1} \times \text{d}^{-1}$  for the digestion configuration, and from  $298.0 \pm 24.5 \text{ NLCH}_4 \times \text{kgTVS}^{-1} \times \text{d}^{-1}$  to  $423.3 \pm 30.9 \text{ NLCH}_4 \times \text{kgTVS}^{-1} \times \text{d}^{-1}$  for the co-digestion one. Furthermore, an additional hydrogen-rich biogas of about  $12.6 \pm 5.0 \text{ NLH}_2 \times \text{kgTVS}^{-1} \times \text{d}^{-1}$  and  $8.6 \pm 4.8 \text{ NLH}_2 \times \text{kgTVS}^{-1} \times \text{d}^{-1}$  was also produced for the digestion and co-digestion configuration respectively. This improvement in bio-fuel production was mainly due to the increase in the feedstocks hydrolysis during the preliminary DF stage [21,25]. Concerning the assessment of the DF effect on digestate stability, Table 4 shows the process performances expressed in terms of biological stabilization and TVS removal efficiency.

**Table 4.** Process efficiency expressed in terms of biological stabilization and total volatile solids (TVS) removal.

Process	Stage	Digestion (FW)		Co-Digestion (FW + WWS)	
		Stabilization (%)	TVS Removal (%)	Stabilization (%)	TVS Removal (%)
One-stage	AD	30.3	67.0	6.5	61.0
Two-stage	DF	−47.4	23.0	−34.3	32.3
	AD	42.2	62.5	55.8	54.5
	DF + AD	14.8	69.4	40.6	71.5

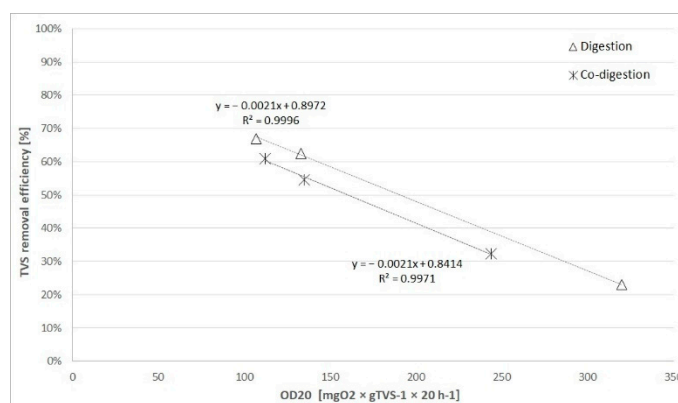
Considering the one-stage system as a reference scenario, stabilization efficiency and TVS removal was assessed for both the digestion and co-digestion process, in order to compare them with the performance obtained with the introduction of DF as a preliminary stage. The stabilization efficiency, which is the estimated decrease of SOUR values in the traditional AD process, is equal to 30.3% and 6.5% for digestion and co-digestion, respectively, with a corresponding TVS removal efficiency of 67.0% and 61.0%.

As concerns the two-stage scenario, process performances were evaluated with reference to the first stage (DF) and the second one (AD). As mentioned above, in the DF stage, a reduction in terms of biological stability was found, due to the increase of the biodegradable matter available in DF digestate, with a resulting increase of SOUR and OD<sub>20</sub> values compared to the input feedstock. Based on this, the stabilization efficiency is a negative value of −47.4% and −34.3% for digestion and co-digestion, respectively. Subsequently, the AD process involves the degradation of the residual organic matter content by raising the biostabilization of the digestate.

Table 4 shows the results of the two-stage scenario expressed both in relation to each individual step of the AD process (in bold) and in relation to the overall process. As expected, the results demonstrate a reduction in stabilization efficiency and a slight TVS removal in the first stage. However, the wide availability of readily biodegradable matter in the DF digestate led to better degradation in the subsequent AD stage, with a stabilization efficiency of about 42.2% and 55.8% for the digestion and co-digestion configurations, respectively. The first value corresponds to a TVS removal efficiency of 62.5% and the second to 54.5%. Concerning the overall performance of the two-stage system, the results are 14.8% and 40.6% in terms of biological stabilization for the digestion and co-digestion scenarios. The best performances found in the two-stage co-digestion process compared to the digestion of the sole

FW were due to the buffer capacity of the WWS present in S2. In fact, the increase in the system's buffer capacity guaranteed maintaining the process stability by counteracting the acidification of the media, due to VFAs production and then also determining preferable performances in terms of stabilization efficiency [60,61]. Based on that, the DF stage seems to influence only the co-digestion system, leading to better results in terms of organic matter degradation. However, the partial results suggest that the introduction of DF as a preliminary stage enhances the performances in the methanogenic reactor due to previous hydrolysis of the substrates.

The results concerning the stabilization efficiency were estimated by the SOUR index, which is directly related to the  $OD_{20}$  index. Figure 4 shows the correlation between TVS removal efficiency and  $OD_{20}$  values. The TVS removal efficiency results are linearly correlated with  $OD_{20}$  ( $r = 0.99$ ) for both the digestion data and the co-digestion one. Since  $OD_{20}$  is the measure of the oxygen consumed by the aerobic microorganism for degrading the organic carbon in a short period of time, high  $OD_{20}$  values correspond to unstable materials that therefore influence the removal efficiency of TVS. The best TVS removal efficiency values were determined for the samples with lower  $OD_{20}$  values and hence, with greater biological stability.



**Figure 4.** Correlation between  $OD_{20}$  and TVS removal efficiency for the digestion and co-digestion process.

#### 4. Conclusions

The co-digestion of FW and WWS demonstrated the best results in the two-stage configuration in terms of biostabilization efficiency. The introduction of the DF stage improved the biological stability of the output digestate as compared to the traditional one-stage AD process. Concerning the overall two-stage process, results showed a decrease in SOUR and  $OD_{20}$  indices, resulting in a stabilization efficiency of 40.6%, which is higher than the 6.5% efficiency found in the one-stage system. The highest biological stability of the digestate coming from the two-stage configuration was due to the better hydrolysis of the substrates, determined by using DF as a preliminary biological treatment for the traditional AD process. Furthermore, the improvement of the biostabilization efficiency in co-digestion was greater than the configuration with the sole FW as substrate, due to the presence of WWS, which improved the buffer capacity of the system and the stability of the process.

In the two-stage system, the enhancement of the VFAs content in the DF digestate provided a large amount of readily biodegradable matter available for the subsequent AD stage. Concerning the stability improvement, evaluated only in relation to the second stage of the process, it reached an efficiency of 42.2% and 55.8% for the digestion and co-digestion respectively, which is higher than that assessed in the conventional process, with values equal to 30.3% and 6.5%. In conclusion, the two-stage system improved the stabilization of the outgoing AD digestate, thanks to the role of DF as a biological pre-treatment that allows better degradation of the substrate, making it more easily degradable for methanogenic bacteria.

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